Virology 432 (2012) 73–80

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Antiviral activity of phage display selected peptides against Porcine reproductive and respiratory syndrome virus in vitro

Ke Liu [a], Xiuli Feng [a], Zhiyong Ma [b], Chao Luo [c], Bin Zhou [a], Ruibing Cao [a], Li Huang [a], Denian Miao [d], Ran Pang [a], Danni He [a], Xue Lian [a], Puyan Chen [a,*]

[a] Key Laboratory of Animal Disease Diagnostic & Immunology, Department of Veterinary Medicine College, Nanjing Agricultural University, YiFu 4037, Nanjing, Jiangsu 210095, China
[b] Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Science, No. 518, Ziyue Road, Shanghai 200241, PR China
[c] Department of Orient Science and Technology College, Hunan Agricultural University, Hunan, PR China
[d] Shanghai Academy of Agricultural Sciences, PR China

ARTICLE INFO

Article history:
Received 2 February 2012
Returned to author for revisions
7 May 2012
Accepted 14 May 2012
Available online 27 June 2012

Keywords:
Porcine reproductive and respiratory syndrome virus
Phage display
Antiviral peptide

ABSTRACT

Porcine reproductive and respiratory syndrome is an important infectious disease of pigs and has a significant harmful effect on the livestock industry, especially in China. PRRSV ORF1b gene encodes primary proteins which play a vital role during PRRSV replication. In this paper, various 12-amino-acid peptides were displayed. These peptides could bind to the polymerase and helicase of PRRSV ORF1b protein, respectively, in which p9 exerted the highest antiviral activity with an IC50 of 56 μM, and the minimum toxicity to cells. It was proved that p9 inhibited PRRSV replication in infected MARC-145 cells in a dose-dependent manner, and the amino acid sequence of HRILMRIR was important for antiviral activity of p9. Also, p9 could bind to the cell membrane and penetrated into cells. These result suggested that p9 might be a potential therapeutic drug for PRRSV infection.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is caused by porcine reproductive and respiratory syndrome virus (PRRSV), and is a widespread infectious disease of pigs (Meulenberg et al., 1993). At the present time, PRRSV is known to damage the immune system of pigs, and the effect of commercial vaccines to prevent PRRSV infection is limited (Christopher-Hennings et al., 1995; Mateu and Diaz, 2008). Therefore, it is urgent to explore new antiviral drugs to control the PRRS.

PRRSV is divided into two major genotypes: the European (EU; type 1) genotype and the North American (NA; type 2) genotype (Meng et al., 1995), which is the main type isolated in China. PRRSV is a single-stranded positive-sense RNA virus of about 15 kB in length. The genome has a cap structure at its 5′-end and a poly(A) tail at its 3′-end and encodes nine open reading frames (ORF1a, ORF1b, ORF2a, ORF2b, ORF3–ORF7). ORF1 and ORF2 encode virus replication-related proteins, and account for 80% of the virus genome (Mounir et al., 1995; Nelson et al., 1995). ORF1a and ORF1b encode a polyprotein, which is proteolytically processed to 12 nonstructural proteins (NSPs). Among these, ORF1b encoded proteins are RNA-dependent RNA polymerase (NSP9), helicase (NSP10) and the conserved C-terminal domain (CTD) (NSP11) (Fang et al., 2004).These proteins are important factors in the process of virus infection (Castel et al., 2011; Sergeeva et al., 2006). Therefore, ORF1b can be used as a potential target for antiviral drug screening.

Phage technology can be used to carry out high-throughput screening of functional polypeptides, and the polypeptide gene cloning number in phage peptide libraries can reach more than a million. This technology has been applied increasingly in drug development trials, and it is an effective method for the development of new drugs (Bai et al., 2006; Hall et al. 2009). At present, many antiviral peptides have been obtained by phage screening technology, such as inhibition polypeptides of infectious bronchitis virus (IBV) (Peng et al., 2006), human immunodeficiency virus (HIV) (Welch et al., 2010), hepatitis C virus (HCV) (Hong et al., 2010), hepatitis B virus (HBV) (Ho et al., 2003), Newcastle disease virus (NDV) and other viruses (Chia et al., 2006). Some of these polypeptides have reached the experimental stage. This experiment uses a 12-peptide phage library to screen 12 peptides for activity against PRRSV ORF1b RNA polymerase and helicase protein. One peptide was found to interact with the PRRSV polymerase and suppress virus replication effectively.

Results

Identification of phage-displayed peptides with PRRSV ORF1B protein

Proteins encoded by PRRSV ORF1b play vital roles during PRRSV replication. To design inhibitors based on these proteins

* Corresponding author. Fax: +86 25 84396335.
E-mail address: Keliu1982@yahoo.com.cn (P. Chen).

0042-6822/$ - see front matter Crown Copyright © 2012 Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2012.05.010

Table 1
List of peptides determined from phage display selection. Peptides P1–P5 are target to Helicase and P6–P14 are target to Polymerase. C1–C5 are sequences determined from *Escherichia coli* control.

Peptide	Target	Amino acid sequence	MW	pI	GRAVY
P1	Helicase	RRRKRPIRRKLR	1691.1	12.78	−2.717
P2	Helicase	HIRLTLSRNKNT	1452.6	12.01	−1.100
P3	Helicase	TRTMTMNQNRRS	1495.6	12.30	−1.925
P4	Helicase	PQTNQTTMKMRM	1466.7	11.01	−1.408
P5	Helicase	IMPTKKIPPIIM	1381.8	10.00	0.708
P6	Polymerase	KKTPPIRIRHRP	1498.8	12.31	−1.750
P7	Polymerase	NIPIKPRPRLMK	1462.8	12.02	−0.867
P8	Polymerase	SPHIIRNHRLSK	1457.7	12.01	−1.100
P9	Polymerase	HRILMRIRQMMT	1586.0	12.30	−0.200
P10	Polymerase	RIIRKSQRSLMN	1501.8	12.30	−0.942
P11	Polymerase	RQPRTPMTRLSR	1498.7	12.48	−1.767
P12	Polymerase	IIRHRSMIITIT	1453.8	12.00	0.833
P13	Polymerase	KTRTMQMRNRMP	1549.8	12.30	−1.808
P14	Polymerase	RMRSKRRKITTR	1588.9	12.60	−2.175
C1	Control	SPRQIMMHMPKP			
C2	Control	QRIRRTPQRPMR			
C3	Control	QMKRMIMMMRRT			
C4	Control	PRPRKMMMMQSK			
C5	Control	HIISIIRKQTRP			

The molecular weight (MW), pI, and grand average of hydropathicity (GRAVY) were predicted with the ProtParam algorithm.

controlling virus replication, phage 12-peptide library was used to screen the peptides binding with ORF1b of PRRSV. After four cycles of selection, a total of 260 phages were selected and sequenced, and 20 peptide sequences were finally obtained by Clustal W screening and BLAST analysis (Table 1).

Inhibitions of peptides on PRRSV replication in vitro

The inhibition ability of synthetic peptides following phage displaying was determined by real-time PCR. MARC-145 cells were inoculated with viruses at 0.01 MOI for 1.5 h at 37 °C, and treated with or without peptides at 500 μM for 24 h, and cells treated with DMSO were used as the negative control. The results showed that p9 peptide exerted the highest antiviral activity among that of peptides with PRRSV polymerase in vitro (Fig. 1).

The results of Real-time PCR and TCID50 showed that p9 was the greatest antiviral peptide among all these selected peptides. Other peptides such as p2, p3 and p13 also showed high antiviral effects. Furthermore, 500 μM p9 exerted higher antiviral activity than that of 10 μM ribavirin (Ibarra and Pfeiffer, 2009).

Antiviral activity of p9

To further investigate the antiviral activity of p9, the toxicities of p9 to MARC-145 cells were detected using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The results showed that CC50 and IC_{50} of p9 (24 h) to MARC-145 cell was 473.00 μM and 56.47 μM, respectively, and CI (CC50/IC50) of p9 was 8.38 (Fig. 2a). When the concentration of p9 was 250 μM, the ratio of virus copy number/cell reference copy number was 0.8625, and cell survival rate was 74.54%. The results of TCID50 are the same as real-time results (Fig. 2b).

To study the time- and dose-dependent effect of p9 on inhibition of PRRSV replication, MARC-145 cells were incubated with p9 from 31.25 μM to 1000 μM for the indicated periods at 37 °C. It has been reported that a complete cycle of PRRSV replication is 24 h (Cafruny et al., 2006). As shown in Fig. 2c, during PRRSV replication (12 h), 1000 μM, 500 μM and 250 μM p9 showed significant inhibition on PRRSV replication. After 24 h, p9 exerted inhibition on intracellular PRRSV numbers of MARC-145 cells in dose-dependent manner. Furthermore, during the second

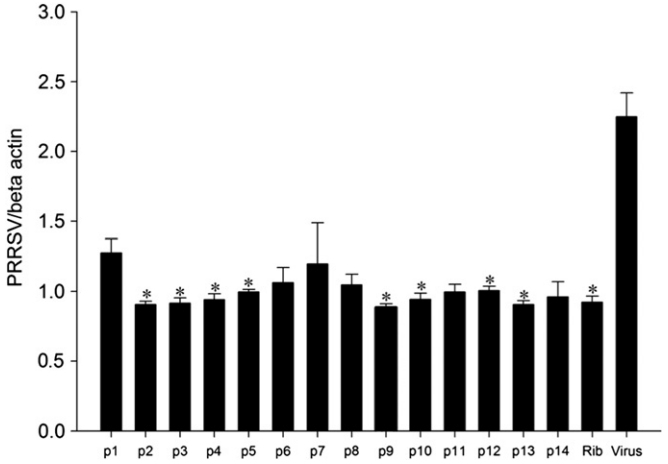

Fig. 1. Peptide inhibition of PRRSV infection in MARC-145 cells at 24 h. Primary screening of antiviral activity of synthetic peptide. Antiviral activity of all peptides was tested by real-time PCR method. Samples were collected at 24 h after virus infection. All experiments were repeated at least three times, and all measurements were made in triplicate, mean ± standard deviation (SD) is shown ($p < 0.05$, indicated by *) p9 IC_{50} and CC_{50}. (B) Antiviral activity of p9 by $TCID_{50}$. (C) Time–effect and dose–effect of p9 P9 inhibited PRRSV by dose-dependent manner. 12 h, 1000, 500 and 250 μM concentration p9 inhibited virus proliferation markedly. 24 h to 48 h, this dose-dependent inhibition was oberversed more markedly. Furthermore, the intracellular PRRSV numbers in p9-treated MARC-145 cells were observed increased in time-dependent manner. (D) Retention period of p9 in MARC-145 by fluorescence. (E) p9 added at 4 h before and after virus.

replication cycle (36 h and 48 h), with increasing doses of p9, the intracellular PRRSV numbers of MARC-145 cells treated with p9 were gradually decreased. Furthermore, cytotoxicity in these two points was very low (data not shown). These results suggested that p9 inhibits PRRSV replication in a dose-dependent manner.

To detect the intracellular retention time of p9 which is a potential important antiviral factor, MARC-145 cells were treated with rhodamine-labeled p9, respectively. Results of light intensity/scanning area of fluorescence gray scanning showed that the retention time of p9 in cells was at least up to 144 h, and at 24 h, the fluorescence intensity of rhodamine-p9 is highest among all indicated times. However, at 48 h and 72 h, the intensities of rhodamine-p9 were decreased, compared to that of 24 h. Subsequently, fluorescence levels were dramatically reduced, and were almost not observed after 96 h (Fig. 2d).

To further study the inhibition effects of p9, p9 was added for 4 h before and after virus infection and at 0 h after virus infection, respectively (Fig. 2e). The results of Real-time PCR showed that at 24 h, the intracellular PRRSV numbers in PRRSV-infected MARC-145 cells pre-incubated with p9 for 4 h or 0 h were dramatically decreased, compared to that of DMSO control. Although PRRSV RNA of MARC-145 cells treated with p9 after PRRSV infection were lower than that of DMSO control, it was significantly higher than that of pre-incubated with p9 for 4 h or 0 h. Also, results at 48 h were similar that of 24 h. These results suggested that p9 stimulation before or together with PRRSV infection could strongly inhibit PRRSV replication in MARC-145 cells.

P9 penetrates cells

As an antiviral peptide, we detected penetration of p9 in MARC145 cells. Results showed p9 could adsorb to cell membranes in a short time (2 h), and could penetrate into the cytoplasm within 24 h. These results were observed in other cells too (data not shown) (Fig. 3).